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Simulated global increases in atmospheric CO_2 alter the tissue composition, but not the growth of some submerged aquatic plant bicarbonate users growing in DIC rich waters

Andreas Hussner[a,b,*], Rosali Smith[b], Tabea Mettler-Altmann[c], Martin P. Hill[b], Julie Coetzee[d]

[a] Photosynthesis and Stress Physiology of Plants, Heinrich-Heine-University, Universitätsstr. 1, D-40225, Düsseldorf, Germany
[b] Centre for Biological Control, Department of Zoology and Entomology, Rhodes University, P.O. Box 94, Grahamstown, 6140, South Africa
[c] Cluster of Excellence in Plant Sciences (CEPLAS) and Institute of Plant Biochemistry, Heinrich-Heine-University, Universitätsstr. 1, D-40225, Düsseldorf, Germany
[d] Centre for Biological Control, Botany Department, Rhodes University, P.O. Box 94, Grahamstown, 6140, South Africa

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ABSTRACT

Current global change scenarios predict an increase in atmospheric CO_2 from the current 380 ppm to a value ranging from 540 ppm to 960 ppm by the year 2100. The effects of three air CO_2 levels (400, 600 and 800 ppm) on five submerged aquatic plants that utilize HCO_3^- were studied, using the elevated CO_2 Open Top Chamber facility at Rhodes University (Grahamstown, South Africa). Plants grew in water with two different initial dissolved inorganic carbon (DIC) concentrations of 1.5 and 3.0 mM. Overall, the growth rates and biomass allocation to roots were not affected by the initial DIC and air CO_2, even though differences between the species were found. Furthermore, no overall effects were found on net photosynthesis, chlorophyll and starch content, even though significant effects of CO_2 and DIC were observed in some species. In contrast, with increasing DIC and air CO_2 a significant global decline in leaf nitrogen content linked with an increased C:N molar ratio was observed. The results indicate that submerged aquatic HCO_3^- users will be less affected by atmospheric CO_2 increases when growing in DIC rich waters, in comparison to obligate CO_2 users growing under CO_2 limiting conditions as documented in previous studies. However, the changes found in plant nitrogen illustrate that atmospheric CO_2 increases will affect nitrogen absorption by submerged plants, with subsequent ecosystem level effects.

1. Introduction

Current global change scenarios predict an increase in atmospheric CO_2 from the current 380 ppm to a value ranging from 540 to 960 ppm by the year 2100 (IPCC, 2007). Terrestrial C_3 plants will benefit from an increase in atmospheric CO_2, even though the effects will differ between species (Long et al., 2004). While plants will show a strong increase in net photosynthesis and growth due to the higher photosynthetic CO_2 fixation rate in the short term, this stimulating effect on growth and photosynthesis decreases in the long-term (Long et al., 2004). In contrast, no direct stimulation of photosynthesis in terrestrial C_4 plants is expected except during drought events (Long et al., 2004). The effects of increasing atmospheric CO_2 on submerged freshwater aquatic macrophytes seem far more complex than previously thought, and might be species and waterbody specific.

In freshwater ecosystems, the availability of inorganic carbon for primary production depends on (i) the dissolved inorganic carbon (DIC) availability and (ii) the water pH, which determines the portion of CO_2, HCO_3^- and CO_3^{2-} within the DIC pool (Pedersen et al., 2013). In general, the DIC and the water pH are predominantly influenced by calcareous and silicate rock weathering, carbon uptake by primary producers, and the CO_2 released by consumers (Pedersen et al., 2013). This can result in a decoupling of the carbonate equilibrium from the partial CO_2 pressure in the atmosphere (Maberly et al., 2013; Weyhenmeyer et al., 2015). Increasing the atmospheric CO_2 will increase the overall DIC availability and decrease the water pH in most aquatic ecosystems. Within a given pH range, this will increase the proportion of CO_2, HCO_3^- or both within the DIC pool (Pedersen et al., 2013).

For submerged vascular plants, CO_2 is the preferred carbon form and a number of species obligately use CO_2 (hereafter CO_2 users). However, about 50% of all submerged aquatic plants have evolved mechanisms to use HCO_3^- (hereafter HCO_3- users) to cope with CO_2 limitation. This additional carbon source is particularly useful since HCO_3^- is the dominant carbon form at pH 6.4–10.3 (Maberly and

* Corresponding author. Present address: Förderverein Feldberg-Uckermärkische-Seenlandschaft e.V., Am Markt 19, D-17268 Templin, Germany.
E-mail address: hussnera@hhu.de (A. Hussner).

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Madsen, 2002; Yin et al., 2017). This ability to use HCO_3^- is caused by morphological and physiological adaptations (Maberly and Madsen, 2002; Bowes, 2011; Eusebio Malheiro et al., 2013; Pedersen et al., 2013; Hussner et al., 2016).

The efficiencies of these HCO_3^- use mechanisms vary largely, but the most efficient (expressed as the ratio of growth under HCO_3^- / CO_2 use conditions) were found in single cell C_4 species like *Hydrilla verticillata* and *Egeria densa* (both Hydrocharitaceae). Other mechanisms such as leaf polarity in *Elodea* spp. and carbonic anhydrases in *Myriophyllum* spp. are less efficient (Prins and Elzenga, 1989; Hussner et al., 2016; Dülger and Hussner, 2017). Interestingly, while the effect of CO_2 enrichment (pH 6.7 and a DIC of 0.85 mM) affected growth and biomass allocation between high and low efficiency HCO_3^- users, we observed stronger effects of CO_2 enrichment on growth and photosynthesis with lower HCO_3^- use efficiency (Hussner et al., 2016; Dülger and Hussner, 2017).

Previous studies have shown that the affinity of submerged aquatic plants to CO_2 and HCO_3^- depends on the species and the growth conditions. Enrichment in CO_2 decreases the affinity for CO_2 in obligate CO_2 users, and decreases the affinity for both CO_2 and HCO_3^- in HCO_3^- users (Sand-Jensen and Gordon, 1986; Maberly and Madsen, 2002; Hussner et al., 2016). Elevated HCO_3^- availability during growth decreases the affinity to HCO_3^- in HCO_3^- users, but this decreased affinity to increased HCO_3^- availability is less pronounced than for increasing CO_2 availability (Sand-Jensen and Gordon, 1986; Madsen et al., 1996). The increased availability of CO_2 during growth decreased the leaf internal nitrogen, chlorophyll and protein content in HCO_3^- users (Madsen et al., 1998; Hussner et al., 2016; Dülger et al., 2017), which is most likely a result of downregulated photosynthesis induced by starch accumulation in the leaves (Dülger and Hussner, 2017; Dülger et al., 2017). Surprisingly, this decrease in leaf nitrogen was accompanied by an increased biomass allocation to roots, and it was hypothesized that the roots serve as storage organs for starch (Eusebio Malheiro et al., 2013; Hussner et al., 2016; Dülger and Hussner, 2017; Dülger et al., 2017).

Nevertheless, photosynthesis in submerged aquatic plants is generally more responsive to increases in CO_2 than HCO_3^- (Maberly and Madsen, 1998). Moreover, submerged aquatic CO_2 users generally have a higher affinity to CO_2 than HCO_3^- users (Madsen et al., 1996; Maberly and Madsen, 1998). These differences in the affinity to CO_2 and HCO_3^- between CO_2 and HCO_3^- users determine the macrophyte community composition and explain differences between, and even within, water bodies (Sand-Jensen, 1989; Maberly et al., 2015; Titus and Pagano, 2017).

Surprisingly, little is known about the potential effects of increasing atmospheric CO_2 levels on aquatic biota in general (Hasler et al., 2016), and submerged aquatic plants in particular, even though species composition is highly related to pCO_2 (Demars and Trémolières, 2009). Due to species-specific responses to increases in CO_2 and HCO_3^- availability, it is highly likely that the effects of increasing atmospheric CO_2 will vary, depending on macrophyte species and water body. In DIC-poor softwater lakes, where the aquatic plant macrophyte community is dominated by plants using CO_2 from the sediment (e.g. *Lobelia dortmanna*, Winkel and Borum, 2009), even small increases in CO_2 availability may increase the likelihood of invasions by water column CO_2 users (i.e. elodeids, Spierenburg et al., 2009). Consequently, DIC-rich waters with high pH (> 8.2), where CO_2 availability is limiting for the growth of CO_2 users (e.g. *Myriophyllum triphyllum*), are dominated by HCO_3^- users (Hussner et al., 2015; Dülger and Hussner, 2017), while increases in CO_2 availability would support the growth and competitive strength of CO_2 users. The lowest effects of increasing atmospheric CO_2 could be expected for high efficient HCO_3^- users in DIC rich (and thus highly pH buffered) waters when limitation of both CO_2 and HCO_3^- occur only at pH values of approx. 10 or higher (depending on the total amount of DIC in the water and thus the CO_2 and HCO_3^- availability at a given pH).

Based on these findings, we hypothesize that in DIC rich waters, the effects of global change CO_2 increases on net photosynthesis and biomass production of HCO_3^- users will be low. In contrast, we expect reduced chlorophyll and nitrogen contents, as the increased availability of DIC in general, and the use of CO_2 in particular, requires a lower investment in carboxylating enzymes and maintenance of proteins than observed in HCO_3^- users (Madsen et al., 1996; Hussner et al., 2016).

2. Material and methods

2.1. Plant material

The experiments were carried out with five submerged Hydrocharitaceae species (*Egeria densa* Planch., monoecious *Hydrilla verticillata* (L.f.) Royle, *Lagarosiphon cordofanus* Casp., *Lagarsiphon major* (Ridl.) Moss and *Lagarosiphon muscoides* Harv.). Plants were taken from stock cultures from greenhouses located at the Waainek Research Facility of Rhodes University, Grahamstown, South Africa. Plants were precultivated in 1000 L tanks filled with tap water containing 1.5 mM DIC under 80% shade cloth. For the experiment, apical shoots of 10 cm in length were cut and the fresh mass of the apical tips was determined after gently blotting dry on soft paper tissue. The dry mass of the apical shoots was calculated based on the mean value of the dry mass to fresh mass ratios which were determined for 5 subsamples of each species.

2.2. Experimental setup

Apical shoot sections were planted into 200 ml pots filled with pond sediment collected from Jameson Dam, Eastern Cape, South Africa (-33.319073 S; 26.444206 N). Each pot was capped with a 0.5 cm layer of washed river sand to reduce nutrient loss into the water column. The sediment naturally contained 11.0 mg N kg^{-1} and 11.65 mg P kg^{-1}. Two pots per species were placed into 18 round 60 L buckets (54 cm in diameter and 31 cm in height), filled with tap water containing 1.5 mM DIC. Two buckets with the full complement of test species were placed into nine outdoor open top CO_2 growth chambers (two buckets per chamber) located at the elevated CO_2 Open Top Chambers facility at Rhodes University, Grahamstown, South Africa.

The CO_2 level in the air was adjusted to 400 ppm, 600 ppm or 800 ppm, replicated three times per CO_2 treatment (Supplemental Fig. 1). In one bucket per chamber, additional inorganic carbon was added to a final DIC of 3 mM to get two different initial DIC levels (1.5 and 3 mM).

In each bucket, the water was mixed with the ambient air using submersible water pumps (Trixie 200, Tarp, Germany). The water pH was measured twice a week with a handheld pH meter (Eutech Elite Pocket pH meter (ThermoFisher Scientific)). Plants were grown for 33 days under 80% shade cloth in the mesocosms with a natural light regime from end of January to end of February 2017, during South African summer.

2.3. Net photosynthesis measurements

On the morning of day 32, one apical shoot of approximately 5 cm in length per plant species in each pot was taken for net photosynthesis measurements. Net photosynthesis was determined after incubation for 45 min in growth chambers at 25 °C and 100 µmol photons m^{-2} s^{-1} in 100 ml stopper bottles. The stopper bottles were filled with tap water, which was used for the growth experiment, containing 1.5 mM DIC. The pH was adjusted to pH 9 (by adding 0.1 mM NaOH) to induce HCO_3-use, and two bottles without plants were used as controls. The dissolved oxygen content of the water was measured using an oxygen electrode

(type OXI-340, WTW, Weilheim, Germany), and compared to the controls. After the measurements were taken, the plants were dried at 90 °C to a constant weight, weighed, and the net photosynthetic rate was calculated on a dry mass (dm) basis.

2.4. Harvest

The plants were harvested on day 33 and subsamples of leaves were randomly taken from the upper 5 cm of the shoots, shock frozen in liquid nitrogen and subsequently lyophilised. The remaining plants were cleaned from sediment, and the roots and shoots were separated. The dry mass of shoots and roots were determined after drying the plant material at 90 °C to a constant weight.

Relative growth rate was calculated using the following equation:

$$\text{Relative growth rate (RGR)} = (\ln W_f - \ln W_i)\ T^{-1}$$

where W_i is the initial total plant dm and W_f the final total plant dm after T = 33 days

The root mass fraction (RMF) was calculated as:

RMF = g dm of roots / g total plant dm

2.5. Determination of C, N, chlorophyll and starch content within leaves

The lyophilised leaf material was ground to a powder using a Mixer mill (type MM301, Retsch, Germany). Subsamples of this powder were used for the analysis of leaf internal C, N, chlorophyll and starch content.

For the determination of C and N, an isotope ratio mass spectrometer coupled to an elemental analyser (type Isoprime 100, Elementar Analysensysteme, Hanau, Germany) was used. The elemental analyser was calibrated every 30 samples to an acetanilide standard (C = 71.09%; N = 10.36%) to assure accuracy.

The chlorophyll content was determined according to Arnon (1949). One to two milligrams of the leaf powder were extracted in the dark at 4 °C for 12 h in 80% acetone. Subsequently, the samples were centrifuged at 15,682 *g* and the chlorophyll content was measured spectrophotometrically in the supernatant using the specific absorption maxima of chlorophyll *a* (663 nm) and chlorophyll b (645 nm).

Starch analysis was carried out following the protocol provided by Smith and Zeeman (2006) and the description of Dülger et al. (2017) and Dülger and Hussner (2017). The base and final absorption of NADP at 340 nm was measured using a 96-well plate reader (Bio Tek Synergy HT, Winooski, USA).

2.6. Statistical analyses

For the overall analysis, we used a three-way ANOVA to test the effects of the treatments, species and their interactions, and data were ln transformed if needed. For each single species, a two-way ANOVA followed by Tukey's HSD was carried out to test the effects of air CO_2 and DIC. All analyses were peformed by using R statistical software version 3.4.1 (R Core Team, 2017).

3. Results

3.1. Water pH and the availability of CO_2 and HCO_3^-

Due to different atmospheric CO_2 conditions, water in the different treatments differed significantly in pH and thus the proportion of CO_2 and HCO_3^-. The lowest pH was found in the 1.5 mM and 800 ppm treatment (8.07 ± 0.03), while the highest (8.63 ± 0.02) was found under 3 mM and 400 ppm (Fig. 1). Consequently, the proportion of free CO_2 within the DIC pool in all treatments ranged between 0.5 and 2.0%, while the majority of the DIC was present as HCO_3^- (97.1–97.5 %).

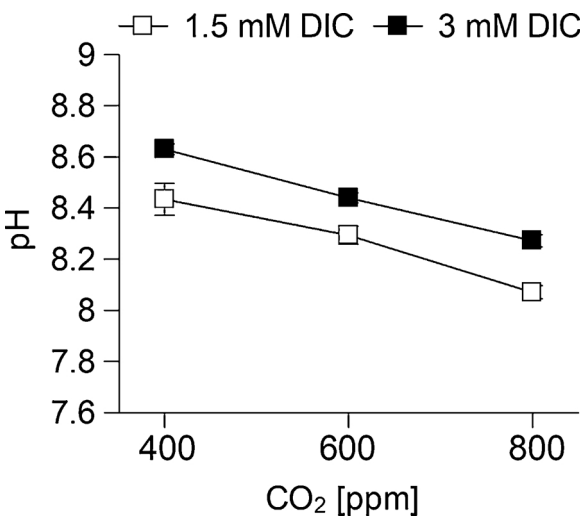

Fig. 1. pH values in the 60 L tanks during the growth period of 33 days at low (1.5 mM) and high (3 mM) DIC levels and at 400, 600 and 800 ppm CO_2 in the air. Data shown as means ± SE.

3.2. Growth and biomass allocation to roots

The growth rates differed significantly between the species (Table 1 and Fig. 2) with *H. verticillata* exhibiting the fastest growth. Overall, plant growth rates were not affected by the DIC and the CO_2 levels in the air (Table 1). However, *L. cordofanus* and *L. major* had significantly higher growth rates at 3 mM DIC, and for both species, an interactive effect for DIC and CO_2 was found (Fig. 2). In contrast to the growth rate, RMF was significantly affected by DIC and differed significantly between the species, while air CO_2 had no effect on RMF. The highest RMF of 0.395 ± 0.022 was found in *L. muscoides* at 3 mM DIC and 800 ppm CO_2, the lowest was found in *L. cordofanus*, with only 0.055 ± 0.012 at 1.5 mM DIC and 400 ppm CO_2 (Fig. 2). In *H. verticillata*, the RMF increased significantly with increasing air CO_2. For *L. major*, a significant interaction between DIC and air CO_2 on RMF was found, when RMF increased with increasing air CO_2 at 3 mM DIC, but decreased at 1.5 mM (Fig. 2).

3.3. Nitrogen and carbon content of leaves

Overall, the nitrogen content of leaves was significantly higher at 1.5 mM than at 3.0 mM DIC. Moreover, the N content significantly decreased with increasing air CO_2 with significant interactions between DIC, CO_2 and species (Table 1, Fig. 3). Similarly, the leaf internal C content was significantly lower at 3 mM than at 1.5 mM, and lower at 800 ppm than at 400 and 600 ppm. The highest C content was found in the 1.5 mM DIC and 400 ppm treatments, and the lowest C content under 3 mM and 800 ppm (Fig. 3, Table 1), with a strong interaction between DIC, CO_2 and species (Table 1). The C:N molar ratio was significantly higher at 3 mM than at 1.5 mM DIC and increased with increasing CO_2 (Fig. 3).

3.4. Net photosynthesis, chlorophyll and starch content

Net photosynthesis was not affected by the growth conditions, but differed significantly between the species (Fig. 4). *L. muscoides* showed the highest rate of photosynthesis, while the lowest rate of photosynthesis was observed for *E. densa*. Similarly, the chlorophyll content was significantly different between the species (Fig. 4), and again *L.*

Table 1
F-values of the three-way Analysis of Variance (ANOVA), significant values appear in bold.

	CO_2	DIC	Species	CO_2 x DIC	CO_2 x Species	DIC x Species	CO_2 x DIC x Species
RGR	0.85	1.66	**13.37*****	0.85	0.69	1.40	2.17
RMF	1.32	**4.13***	**23.83*****	2.90	0.84	1.28	0.86
N	**6.69****	**4.79***	2.04	**11.78*****	**3.29****	**4.43****	**4.39*****
C	**9.91*****	**19.29*****	**10.79*****	**10.99*****	**7.17*****	**14.64*****	**7.62*****
C:N	**3.63***	2.32	1.17	**6.46****	**2.51***	**3.13***	**3.50***
Chl_{tot}	1.53	1.07	**3.93****	0.37	1.18	1.24	1.56
Starch	2.14	0.08	2.34	1.49	**2.87****	1.77	1.32
PS	0.74	0.75	**25.09*****	1.96	0.39	0.92	0.49

muscoides had the highest chlorophyll content. Even though View Commentsthere were no overall effects of air CO_2 and DIC, the chlorophyll content decreased significantly for *H. verticillata*, *L. cordofanus* and *E. densa* with increasing air CO_2, while the chlorophyll content in *L. muscoides* was significantly higher at 1.5 than at 3 mM DIC. For the starch content, only an interactive effect of air CO_2 and species was found Fig. 4.

4. Discussion

Several studies report the possible effects of increasing CO_2 and HCO_3^- availabilities on submerged plant growth (Olesen and Madsen, 2000; Eusebio Malheiro et al., 2013; Hussner et al., 2015) and community composition (Maberly et al., 2015; Titus and Pagano, 2017). In contrast, our study, which focused on HCO_3^- users only, found no effect of increasing air CO_2 on submerged aquatic plant growth.

DIC limited freshwater systems like softwater lakes are naturally dominated by isoetids acclimated to DIC and CO_2 limitation (Winkel and Borum, 2009). In these systems, increasing CO_2 availability enhances growth and increases the likelihood of invasions by elodeids (Spierenburg et al., 2009, 2010). In contrast, the effects of increasing air CO_2 in DIC rich waters will be lower or even absent, when plants are not limited by CO_2 or HCO_3^-.

In general, obligate CO_2 users have a higher affinity to CO_2 than HCO_3^- users and will thus become saturated at lower CO_2 levels (Maberly and Madsen, 1998). But in most freshwater systems, the amount of CO_2 is much lower than the availability of HCO_3^- due to the pH dependent partioning of CO_2 and HCO_3^- within the DIC pool (Pedersen et al., 2013). In comparison to CO_2, the HCO_3^- saturation point differs between CO_2 and HCO_3^- users and even between HCO_3^--users, due to the different affinities of the species to HCO_3^- (Hussner et al., 2016). It makes sense that the effects of increasing air CO_2 will decrease with increasing affinity to HCO_3^- of the species and the overall DIC availability within the water.

The highly effective HCO_3^- uptake of *H. verticillata* and *E. densa* (Hussner et al., 2016) showed no differences in RGR, indicating that both species were saturated in their carbon uptake in all treatments in our study. For the two species with the lowest growth rates in our study (*L. cordofanus* and *L. major*), a significant effect of DIC on RGR was found, indicating that both species were not carbon saturated at 1.5 mM DIC. However, the response in RGR with respect to increasing air CO_2 was still low. This was most likely due to the elevated pH in the test systems. Because of this high pH and consequently a high proportion of HCO_3^-, the increase in air CO_2 did not increase the amount of free CO_2 in the water markedly (Pedersen et al., 2013). Moreover, the effect of increasing CO_2 is generally higher in plants restricted to CO_2 use, and becomes less with increasing ability to use HCO_3^- in HCO_3^- users (Hussner et al., 2016). Similarly, the small differences in the CO_2 availability (from 0.5 to 2.0 % of the total DIC) might also explain why no effect of increasing air CO_2 on the RMF was found. Corresponding increases in CO_2 availabilty and RMF have been previously documented, but this occurred when CO_2 increased by the factor 10–20

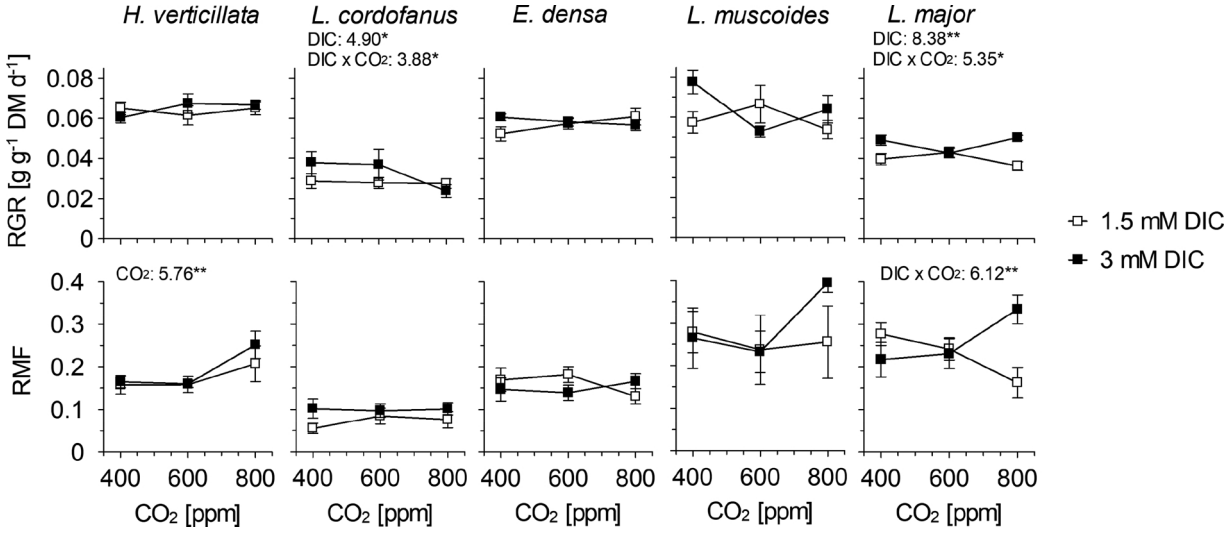

Fig. 2. Relative growth rates (RGR) and root mass fractions (RMF) of the plants after a growth period of 33 days at low (1.5 mM) and high (3 mM) DIC levels and at 400, 600 and 800 ppm CO_2 in the air. Means ± SE of 5–6 replicates are shown. The *F*-values and level of significance for differences between the different DIC concentration (DIC), between the air CO_2 levels (CO_2) and their interaction (DIC X CO_2) are given from two-way ANOVA (significance levels: *P < 0.01; **P < 0.001; ***P < 0.0001).

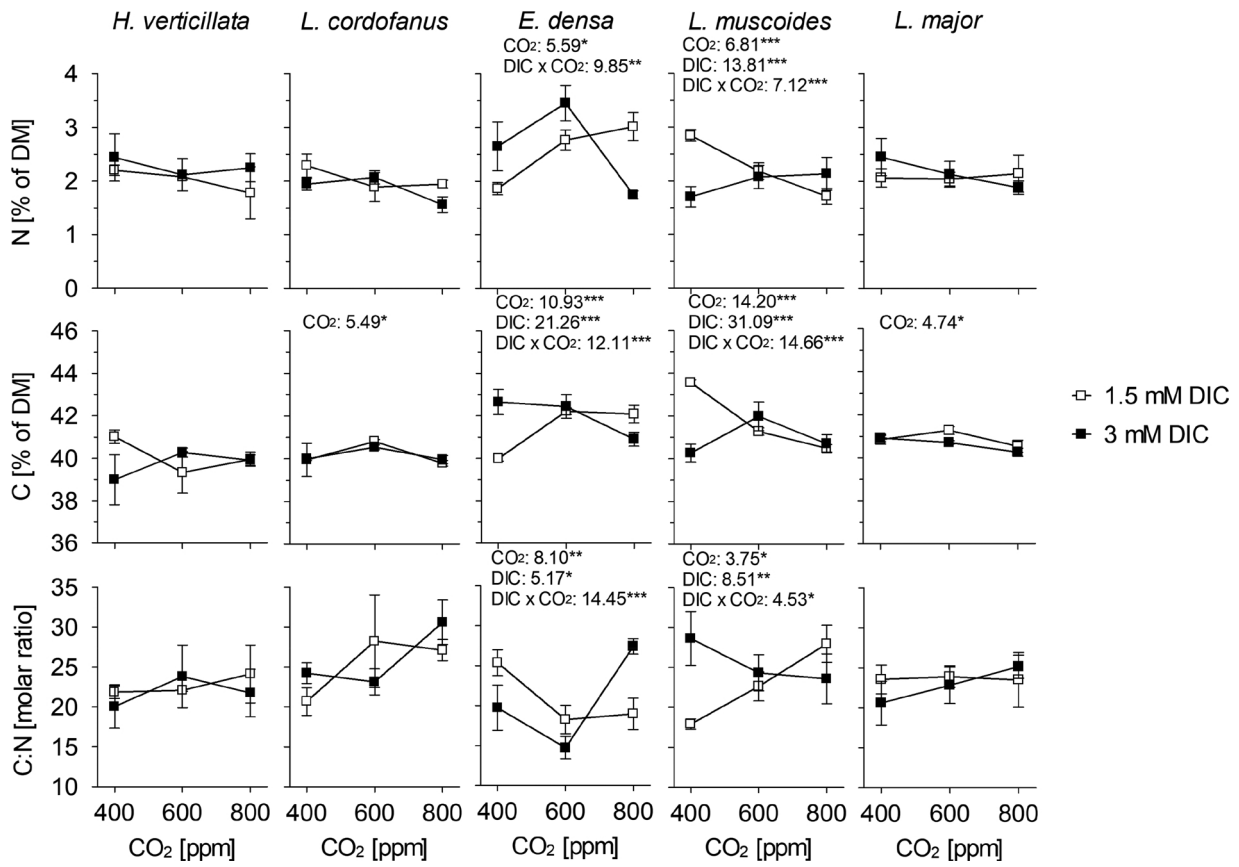

Fig. 3. The leaf nitrogen and carbon content and the C:N molar ratio after a growth period of 33 days at low (1.5 mM) and high (3 mM) DIC levels and at 400, 600 and 800 ppm CO_2 in the air. Means ± SE of 3–4 replicates are shown. Probabilities and *F*-Values as in Fig. 2.

(Hussner and Jahns, 2015; Dülger et al., 2017), and was more pronounced in plants with no or low HCO_3^- use capacity (Hussner et al., 2016; Dülger and Hussner, 2017). The high RMF found at 3 mM DIC indicates that both CO_2 and HCO_3^- availability may affect the RMF, even though the effects of increasing HCO_3^- will be limited to HCO_3^- users.

Decreasing N content of submerged plants has been found in previous studies where increased CO_2 availability led to a lower investment into carboxylating enzymes (Hussner et al. 2016, Dülger et al., 2017). The current experiment further validates this observation. This might explain why submerged aquatic plant species growing in DIC rich and predominantly eutrophic conditions, are able to become invasive even under oligo- to mesotrophic conditions, like *Ceratophyllum demersum* in DIC rich Lake Tarawera, NZ (Wells et al., 1997; Timperley and Vigor-Brown, 1986). Consequently, any changes in DIC or CO_2-availabilities may affect the nutrient cycle in freshwater ecosystems due to changing N absorption of submerged plants – a consequence which has barely been studied to date.

Prior studies documented that starch enrichment in submerged aquatic plants under CO_2 enrichment is most pronounced under N limiting conditions and is higher in CO_2 users than in HCO_3^- users (Dülger et al., 2017). Thus the high nutrient content of the sediment used in this study resulted in generally high accumulation of N within the leaves, which was in most cases much higher than the 1.82% Demars and Edwards (2007) calculated as a 95% growth rate threshold for submerged plant species. This high nutrient sediment may have strongly limited the effects that CO_2 and DIC enrichment otherwise might have on the internal starch content. Additionally, as the starch content is significantly correlated with the chlorophyll content in submerged aquatic plants (Dülger and Hussner, 2017; Dülger et al., 2017), only minor effects of DIC and CO_2 enrichment on chlorophyll content were found.

To summarize, the present study illustrates the varying effects that CO_2 and DIC enrichment might have on submerged aquatic plant species. In our study, efficient HCO_3^- users were barely affected by increasing CO_2 in the air, while other studies observed much stronger effects on obligate CO_2 users. These differences highlight the species-specific consequences of CO_2 and DIC increase in freshwater habitats.

In addition to changes in atmospheric CO_2 levels, climate change will also cause increasing temperatures (IPCC, 2007), which will reduce the CO_2 absorption by the water column. Moreover, elevated temperatures will in a given range cause an increasing CO_2 and HCO_3^- demand by primary producers, which might limit the effects of the atmospheric CO_2 increase on submerged plants.

Consequently, we conclude that the findings of this and other studies on the effects of increasing CO_2 and HCO_3^- are limited to experimental abiotic conditions, including the proportional availability of CO_2 and HCO_3^-. Any major changes in the availability of CO_2 and HCO_3^- within the experimental setup may lead to different experimental outcomes. Moreover, variation of other abiotic factors like nutrient availability and temperature might lead to different results. However, such studies are strongly required to solve the puzzle of interactive effects of abiotic parameters on submerged aquatic plant growth and physiology.

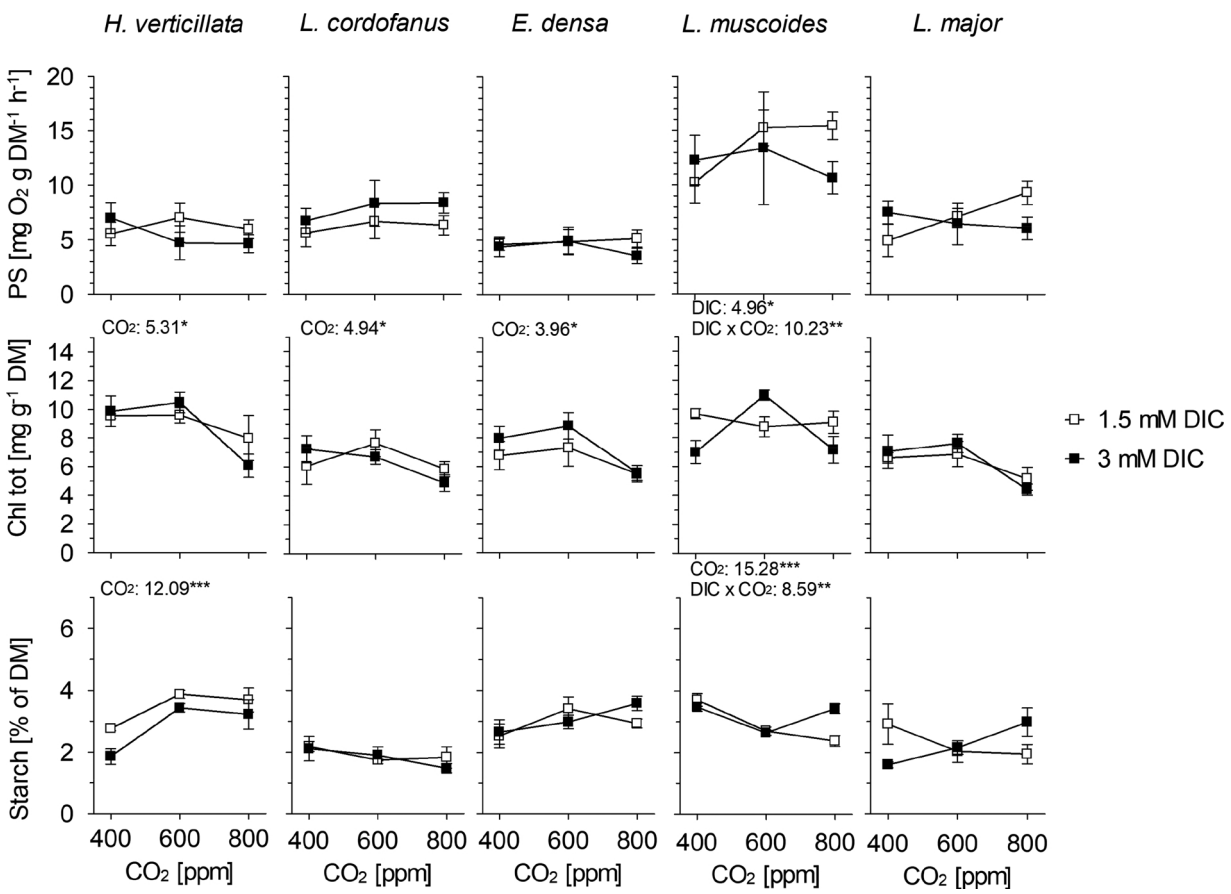

Fig. 4. Net photosynthetic rates (measured at pH 9 at 1.5 mM DIC; n = 5–6), chlorophyll and starch contents (n = 3–4) of the plants after a growth period of 33 days at low (1.5 mM) and high (3 mM) DIC levels and at 400, 600 and 800 ppm CO_2 in the air. Means ± SE are shown. Probabilities and *F*-values as in Fig. 2.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquabot.2018.11.009.

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